Effect of selected weeds of Rajasthan for anti - biofilm screening and its combinatorial efficacy with antibiotics

Research Protocol

For Registration in Ph.D. Programme

to

JECRC UNIVERSITY, JAIPUR

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Faculty of Science

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CERTIFICATE

This is to certify that Research Protocol entitled "…Effect of selected weeds of Rajasthan for anti - biofilm screening and its combinatorial efficacy with antibiotic By Ritu Sharma submitted in fulfilment of the requirement for registration in Ph.D. Program in DEPARTMENT OF BIOTECHNOLOGY under Faculty of Science of JECRC University, Jaipur, during the academic year 2022, is a bonafide record work carried out under Our guidance and supervision.

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INTRODUCTION

A Weed is an undesirable plants that grows in the gardens, lawns and crop fields. They take the nutrients which are essential for the main crops and prevent other plants from growing properly. There is no botanical consequences of this term 'weed' because a plant that is weed in one surrounding may not be consider as weed when growing in a situation where it is needed. (Sahrawat et al.,2020) Some weeds are harmful and poisonous, increase production costs, obstructs irrigation after growing illimitably while many of the weeds have medicinal importance.(Clements and Jones, 2021) Such weeds can be collected from crop fields and used for curing the diseases and any other useful product like bio-pesticides. In India many of the weeds are used as food like Bathua and Chirchita in Ayurveda. Weed plants grow on their own, without any human interferences.

Most of the weed plants have high growth rate with competitive ability. A large proportion of weeds are self -fertilized. Breeding system predicted to have higher colonization ability include hermaphrodites with male and female organs on the same flower and monoecious plants with organs on separate flowers on the same plant. High level of seed production with effective dispersal help them to form a colony. Hybridization with another species results in new genetic combinations which enhance the chance of success when invading in new areas. Many weeds shows resistance or tolerance for herbicides due to their chemical compositions. All these characteristics of weed plants help them to survive in extreme environmental conditions. (Sindhu et al., 2010)

Dandelion (*Taraxacum officinale*)
Crabgrass *(Digitaria saninalis)*
White clover (*Trifolium repens)*
Giant Ragweed *(Ambrosia spp.)*
Plantain Plants (*Plantago major)*
Wild Maddar (*Galium mollugo)*
Clover leaf (*Trifolium)*
Hedge Blindweed *(Convolus arvensis)*
Golden crown beard (*Verbisina encelioides)*

Are some examples of weed plants in India .Some weed plants parts are used in herbal medicines due to their chemical compositions for e.g. The extracts of verbisina encelioides showed the presence of flavonoids, carbohydrates, saponins, phenolic compounds, and sterol in alcoholic extract. (Sindhu et al.2020).

Natural products, such as plants extract, either as pure compounds or as standardized extracts, provide limitless possibilities for new drug findings because of the of chemical assortment. Due to an increasing demand for chemical

assortment in screening programs, seeking therapeutic drugs from natural products, interest particularly in edible plants has grown throughout the world.(Das et al.,2016)

A biofilm forms when defined microorganisms (e.g. some type of bacteria) attach to the surface of some object in a moist environment and began to multiply. Biofilms are microbial communities of surface-attached cells enclosed in extracellular polymeric substances (EPS), which is self- produced. The major feature of microbial biofilms is that the bacteria lives in microenvironment as long as the conditions are favourable. Biofilms have been found to be involved in 70 to 80 percent of all microbial infections in the body. Biofilm associated microbial infections include urinary tract infections, catheter infections, formation of dental plaque, gingivitis and cystic fibrosis. For homogenous biofilm development, the microorganism efficiently attaches itself to a substratum for growth and development processes. (Sahrawat et al., 2020)

Therefore we will try to explore different extracts of selected weed plants of Rajasthan for anti -biofilm screening.

AREA OF STUDY

The arid zone of India covers about 12% of the country's geographical area. A total of 32 million ha is hot arid and is spread over parts of **Rajasthan (61%), Gujarat (20%), Andhra Pradesh and Karnataka (10%), and Punjab and Haryana (9**%). Rajasthan, is the largest state of India and it is situated in the north western region of the country, Jaipur also known as Pink city is located in the north eastern part of the state, is the capital of Rajasthan . Bikaner, Ajmer, Kota, Udaipur, Jodhpur are some of the major cities of Rajasthan. In present study our focus will be Jaipur in and around area.

LITERATURE REVIEW

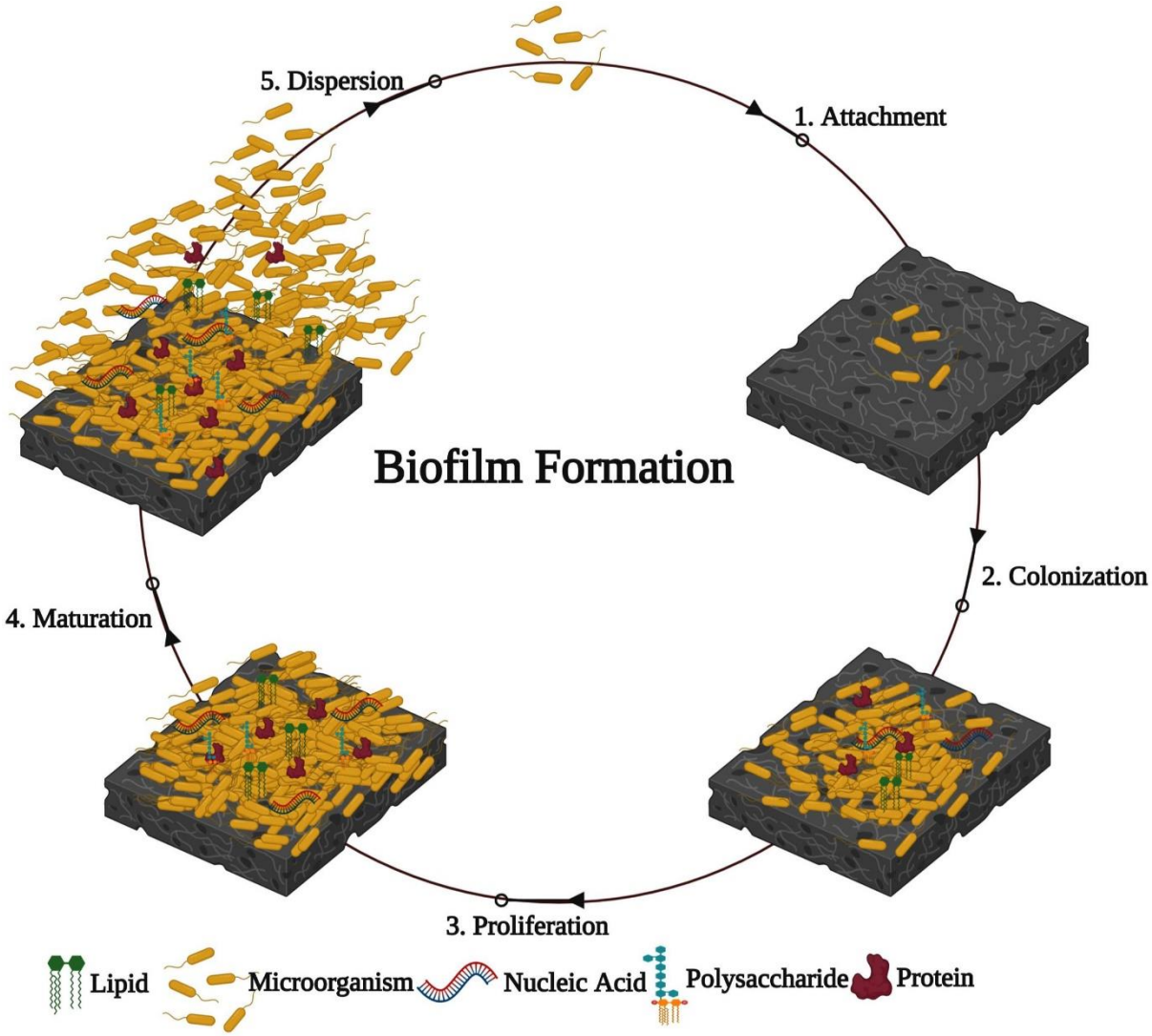
Weed are defined as plants, usually wild or feral that grow and reproduce aggressively. Weed plants considered to be a hindrance in lawn, garden, and crop fields as they are clumsy or prevent the normal growth of main plants as they compete with them for natural resources.

Despite of being harmful some weed plants are beneficial also as they are edible, use for food or herbal medicine e. g. Datura stramonium -Its Leaves and seed contain the alkaloids hyoscine and atropine and are crushed and mixed with ghee to make an ointment for treatment of ringworm. Ocimum spp. - Widely use as medicinal plants to remedy fevers and the whole plant of infusion of the leaves is used as a mosquito repellent.(Alka et. al., 2020) Herbal products are complex mixtures of organic chemicals that may come from any raw or processed part of a plant, including leaves, stems, flowers, roots, and seeds. (Bent, 2008) . Many plant-based products has been observed to be useful to cure various ailments, as compared to conventional therapy. The bioactive compounds extracted from the aerial parts of many plants found in arid zone have anti -biofilm activity against different biofilm-producing bacterial strains. (Lahiri et. al. 2019)

According to the omniscient encyclopaedia Wikipedia a biofilm is “a structured community of microorganisms encapsulated within a self-developed polymeric matrix and adherent to a living or inert surface. (Costerton et al. 1987) indicates that in their natural environment, over 99% of microbes live in micro-ecosystems as biofilms. Biofilm formation is a survival strategy, microbes adopt to enable them survive unpredictable environmental stressors such as temperature changes, desiccation, ultraviolet radiation, cleansing agents such as biocides and disinfectant pressure as well as host immune systems.(Berhe et. al. , 2017)

Development of biofilms is multi step process. (1) Attachment - It starts with the initial adherence of bacteria to the substratum and (2) Colonisation –bacterial pathogen irreversible attachment followed by their colonization in which modification in genes/proteins expression occurs followed by exponential growth phase. (3) Proliferation –The multi-layered bacterial cells accumulated and the exopolysaccharides (EPS) and water channels formation occur, (4) Maturation - the attached multi layered bacterial cells grown into the matured biofilm
(5) Dispersion - facilitating nutrient supply which leads to the maturation of the biofilms. Ultimately surface detachment of the cells starts in the environments

which again restart/recycle the biofilm formation onto the new surfaces. (Sharma et al., 2019)



(STEPS OF BIOFILM FORMATION)

Source – (Srinivasan et al.2021)

Biofilms are a colony of microorganisms in which microbes produced extracellular polymeric substance such as polysaccharides, proteins , DNA, RNA .Water is the main component of biofilm, distributed in a non-homogenous pattern and essentially responsible for the movement of nutrients inside the biofilm matrix (Batoni et al., 2016; Nazir et al., 2019).

EPS is an adhesive that mainly responsible for the biofilm communities slimy nature and is a complex mixture of biopolymers, including polysaccharides,

proteins, e-DNA, and phospholipids (Nazir et al., 2019). In general, EPS composition depends on the type of pathogens, biofilm age, and environmental conditions like temperature, desiccation, oxygen, nitrogen, ph and availability of nutrients.(Mayer et al., 1999; Kostakioti et al., 2013). Another necessary component of extracellular polymeric substance is e-DNA, which enhances biofilm structural integrity, exchange of genetic information, biofilm stability, nutrient provisions and drug resistance (Donlan, 2002; Martins et al., 2010).

The level of health services has advanced greatly over the years, but bacterial biofilm infections are still continuing as a significant risk to public health. Hoiby, Lam and his colleagues were the first to identify a direct correlation between the formation of biofilms and recurrent infections, especially with Pseudomonas aeruginosa in cystic fibrosis patients (Hoiby, 1977; Lam et al., 1980).

Chronic Sinusitis , Ear Infection, Chronic Wounds , Billiary tract infections , Bloodstream infections , Dental plaque, Cystic fibrosis infection , UTI (Urinary tract infection)are various type of tissue related biofilm infections caused by bacterial pathogens. For e g. P. aeruginosa is the main pathogen in the lungs of patients with cystic fibrosis (CF). Chronic colonization by this bacteria leads to lung damage and in the end cause respiratory failure and death in most CF patients.

The genetic and molecular basis of biofilm formation in staphylococci is multifaceted. The ability to form a biofilm presents at least two properties: the adherence of cells and accumulation.

Dental biofilms (plaque), are probably the most common natural biofilm found in humans. This coating is called as acquired pellicle. Then the primary colonizers, first streptococci and later actinomycetes, colonize the surface of the teeth by adhesion molecules and pilli.

Infection or disease	**Common biofilm bacterial species**
Dental caries	Acidogenic Gram-positive cocci (e.g Streptococcus)
Meliodiosis	Pseudomonas pseudo mallei
Biliary tract infection	Enteric bacteria (e. g., Escherichia coli)
Native valve endocarditis	Group A Streptococci
Osteomyelitis	Various bacterial and fungal species – often mixed
Cystic fibrosis pneumonia	P. aeruginosa and Burkholderia cepacia
Musculoskeletal infections	Gram-positive cocci (e.g., Staphylococci)

All these are some human infections involving Biofilm. (Aparna et al., 2008)

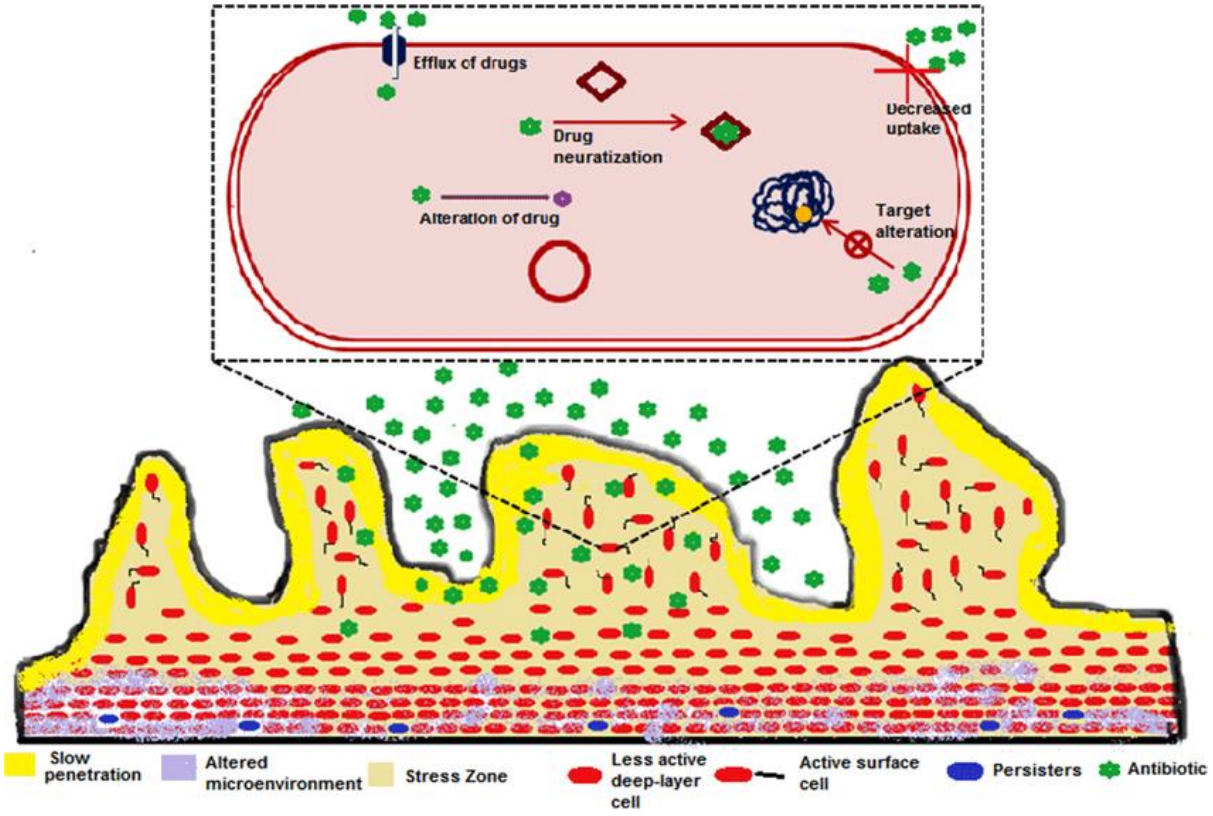
The bioactive compounds present in different plant parts of medicinally used weeds at arid zone have the ability to influence the physiological and cellular activities in animals and humans that consume them. The herbal extracts contain glycoside hydrolase enzyme that helps in degrading the polysaccharide chain by breaking them into smaller subunits or monomers which in turn will help in reducing the biofilm. The a-amylase can cleave the a-1,4-straight-chain linkage and b-1,4 bond and b-1,3 galactosidase can hydrolyse the b-1,3 linkage degrading the external coating (Fleming et al. 2017)

Table - Comparative analysis of mechanism of action of phytochemicals and antibiotics

Sl. no.	Bioactive compound	Phytochemicals	Mechanism of action	Similar action through antibiotics	References
1	Flavonoid	Amentoflavone	Induction of fungal apoptosis	apoptosis Neomycin/polymyxin sulfamethoxazole-trimethoprim (Bactrim Bactrim DS, Septra)	Hwang et al. (2012)
2	Alkaloid	Squalamine	Disintegration of cell membrane due to electrostatic interaction with the cell surface receptors	Imipenem/cilastain (Primaxin)	Salmi et al (2008)
3	Polyphenol	Curcumin	Formation of pore on membrane, ROS, inhibition of morphogenetic switch	Clindamycin (Cleocin)	Lee and Lee (2014), Sharma et al. (2010)
4	Lectins and polypeptides		Interaction with cell adhesion receptors, formation of ion channels in the membrane	Amoxicillin/clavulanate (Augmentin)	Upadhyay and Upadhyay (2014)
5	Saponin	alpha-Tomatine	Induction of fungal apoptosis, disruption of cell membrane		Hwang et al. (2012)
6	Essential oils	Eugenol	Membrane potential disbalance, disintegration of cell membrane	Polymyxin **B**	Zhang et al. (2017)

7	Tannin	Cyanidin	Prevent biofilm formation on the polystyrene surface	Telithromycin	Al-Majedy et al. (2016)

Alkaloids: A vast category of plant secondary metabolites falls under alkaloids. Antimicrobial and anti -biofilm properties of alkaloids have been investigated in different sub-classes like quinolone, agelasine, indolizidine, isoquinoline etc. Essential oils: Like alkaloids, essential oils also fall under the vast category of plant-derived compounds. . Compounds like carvacrol, thymol etc. can cause severe damage to the envelope of both Gram-positive and Gram-negative bacteria and inhibit growth (Nazzaro et al. 2013).



Sources – (Sharma et al.2019)

(Diagrammatic representation of the potential mechanisms of antibiotic resistance in biofilms communities)

Antibiotics are rendered ineffective when biofilms form due to their relative impermeability, the variable physiological status of microorganisms, and subpopulations of persistent strains. Antibiotics resistant state of the biofilm cells lead to a treatment complications in the series of human infections. Bioactive compounds which are present in extracts of some pants in arid zone are called secondary metabolites. These secondary metabolites can enhance the anti-biofilm

activity of extracts and help to prevent innate and acquire biofilm formation when we use it in combination with different antibiotics. Thus here we are using selected weeds of Rajasthan for anti - biofilm screening and its combinatorial efficacy with antibiotics. (LAHIRI et al.,2019)

Objective of the Study

To conduct a survey of traditional medicinal weeds of arid zone.

To enlist and process the selected plants with respect to their ethno medicinal properties.

To analyse the phytochemical properties and anti -biofilm screening of the selected weed plants.

To study the combinatorial effects of selected extract of arid zone plant with selected antibiotics.

To perform the bio efficacy testing of selected plants.

METHODOLOGY

MATERIAL AND METHODS-

Instruments will be used in this study like spectrophotometer (U.V., visible), water bath, electronic weighing balance, distil water assembly, dryer/ oven, vacuum desiccators, sieve, centrifuge, shaker, thermostat , Nutrient agar media, pre-autoclaved antibiotic preparing discs, conical flasks, incubator, forceps , autoclave, and inoculating loop, Erlenmeyer flask, rotary orbital shaker.

COLLECTION-

Samples survey will be collected and authenticated from various regions of Rajasthan.

IDENTIFICATION AND PROCESSING OF PLANT MATERIALS-

All the samples will be authenticated by Herbarium , university of Rajasthan.

EXTRACTION-

Reflux of the plant material (1 kg) with ethanol will be performed for 18 hours. Then filtration will be done, the filtrate will be concentrated to dryness and will be utilised for column chromatography. At the same time each plant material weighing about (100 g) will also be soxhlet extract successively in petroleum, ether, chloroform and alcohol for 6 hours. The extract obtained will be filtered then evaporated to dryness and weighed. The dried extract of each plant material will be dissolved in 10 ml to make a concentration of 1 mg/ml for performing TLC (Thin Layer Chromatography) and HPLC (High Pressure Liquid Chromatography studies).

Microbial strain and Growth Media-

Selected microorganisms (i.e. Pseudomonas, Staphylococcus, E coli and Bacillus) will be used in the study and bacteria will be cultured in tryptic soy broth (TSB) media (Himedia). Initially bacteria will streaked from −80 °C glycerol stock onto tryptic soy agar (TSA) plate and single colony will inoculated into TSB media and incubated at 30 - 37 °C for twenty four hours. From there, bacterial cell suspensions

will be taken for all subsequent experiments. All the experiments will be replicated for statistical analysis using ANOVA or else. Minor modifications will be done as per the environmental conditions of laboratory.

Determination of minimum inhibitory concentration (MIC)-

MIC of azithromycin, gentamicin, against selected micro – organisms (i.e. Pseudomonas, E coli, Bacillus) will be determined by using standard broth micro dilution assay. Experiments will be performed in triplicate.

Evaluation of growth curve-

The growth curve of selected microorganisms (i.e. Pseudomonas, E coli and Bacillus) will be cultivated in the presence and absence of sub-MIC dose of azithromycin and sub-MIC dose of gentamicin with respect to untreated control will be used for studying antibacterial activity. Briefly, standardized test inoculums (10µl) will be added to the wells of 96 well microtiter plate already containing sub-MIC dose of extracts in selected quantities. The plates will be incubated at 30 - 38°C and the OD was recorded at 590nm at twelve hours intervals up to forty eight hours. All the experiments will be replicated for statistical analysis using ANOVA or else. Minor modifications will be done as per the environmental conditions of laboratory.

Evaluation of biofilm forming capability of selected microorganisms (i.e. Pseudomonas, E coli and Bacillus) by staining with Safranin –

Before starting anti-biofilm assay, the selected organisms (i.e. Pseudomonas, E coli, Bacillus) will be tested for its capability to form biofilm. Selected microorganisms will grow in sterile test tubes containing TSB at 30- 37 °C for forty eight hours. After the incubation time, the cultures will be removed and the formed biofilm will be washed three times with sterile phosphate buffer saline (PBS) and stained with 0.1% (v/v) Safranin for 10min. The excess stain will be removed by washing with sterile PBS and dried overnight at 30-38 °C. Safranin from stained adherent selected microorganisms will be re-dissolved in 30% (v/v) glacial acetic acid and measured absorbance at 492nm by UV-Vis spectrophotometer (Parkin-Elmer). The experiment will be performed in triplicate. . All the experiments will

be replicated for statistical analysis using ANOVA or else. Minor modifications will be done as per the environmental conditions of laboratory.

Determination of minimum biofilm eradication concentration (MBEC) -

Bio -film which will be formed as described above and then will transfer it to a standard 96-well plate for MBEC assay as described previously by Wu et al. 2014 with minor modification. Briefly, biofilm will be incubated overnight at 30-38 °C with sub-MIC doses of selected extracts of arid zone plants alone and in combination with other antibiotics. Wells will be rinsed with PBS, and will be placed in a second 96-well plate containing selected extracts of arid zone plants alone and in combination with other antibiotics. Plates will be incubated once again at 30-38 °C for 24h. Then viability of biofilm will be determined by measuring the turbidity at 650nm in a 96-well plate reader (Synergy H1 Hybrid). The minimal biofilm eradication concentration (MBEC) will be defined as the minimal concentration of compound require to eradicate the biofilm. All the experiments will be replicated for statistical analysis using ANOVA or else. Minor modifications will be done as per the environmental conditions of laboratory.

Anti -biofilm formation activity measurements of selected extracts of arid zone plants alone and in combination with either azithromycin or gentamicin-

Interference of biofilm formation upon treatment with selected extracts of arid zone plants alone and in combination with either azithromycin or gentamicin will be performed as the method described above. Sub-MIC doses of selected extracts of arid zone plants alone (150 μg/ml, 130 μg/ml, 110 μg/ml, 90 μg/ml, 70 μg/ml and 50 μg/ml) and in combination with sub-MIC dose of azithromycin (13.75 μg/ml) or gentamicin (2.5 μg/ml) will directly added to bacterial suspension and will be incubated at 30-38 °C for 48h. All test tubes (treated and untreated) will then wash, stain with safranin as per the biofilm forming assay and absorbance (OD) will be measured at 492nm . To find out percentage biofilm inhibition in all treated tubes with respect to untreated control, the following formula was used:

$$\text{Biofilm Inhibition (\%)} = \{(\text{OD of untreated control}) (\text{OD of treated sample})/ (\text{OD of untreated control})\} \text{ X } 100$$

All the experiments will be replicated for statistical analysis using ANOVA or else. Minor modifications will be done as per the environmental conditions of laboratory

Estimation of biofilm total protein concentration-

Concentration of extractable protein will be determined as the measure of selected microorganisms (i.e. *Pseudomonas, E coli and Bacillus*) biofilm population density on a test tube surface (treated and untreated). The microbial population density in biofilm is directly proportional to the amount of EPS. Therefore, to estimate the total extractable protein from tube surface, selected microorganisms will be inoculated at 30-38 °C for forty eight hours with or without sub-MIC doses of selected extracts of arid zone plants alone and in combination. After incubation, we will remove planktonic cells, adhered cells in biofilm will be washed gently with sterile PBS and will be boiled for 30min in 5 ml of 0.5N NaOH (Himedia, India) to extract protein. After that, the suspension will be centrifuged at 10,000rpm for 5min and the resulting clear supernatant will be collected. Supernatant protein concentration will be measured according to Lowry's method. All the experiments will be replicated for statistical analysis using ANOVA or else. Minor modifications will be done as per the environmental conditions of laboratory

Microbial adherence on glass surface analysis by microscopy -

To examine the effect of selected extracts of arid zone plants on biofilm formation over the glass surface, selected microorganisms will separately inoculated in presence and absence of selected sub-MIC doses of selected extracts of arid zone plants alone and in combination with the antibiotics (azithromycin and gentamicin). Bacteria will be grown into 35× 10mm Petri dish containing sterile glass cover slips and incubated at 30-38 °C for 48h. After incubation, cover slips will be collected from each Petri dish, will be washed gently with sterile PBS and will be stained with acridine orange (4 μg ml−1) for 15min in dark. Cover slips will be allowed to dry and live cells in biofilm form attached on glass surface will be visualised under fluorescence microscope (Leica DM 4000B, Germany) 19. Images will be captured from 20 different fields. All the experiments will be replicated for statistical analysis using ANOVA or else. Minor modifications will be done as per the environmental conditions of laboratory

Extraction and measurement of exopolysaccharide (EPS) -

EPS will be measured as described previously by Tribedi and Sil. The biofilm formed on the glass surface will be extracted in sterile water and the suspension will be centrifuged at 3500 g for 20 min at 4 °C. The supernatant will be collected and pellet will be treated with 10mM/l EDTA, vortexed for 15min and will be re-centrifuged to extract cell-bound EPS. The supernatant will be collected and mix with the previous supernatant. The pooled supernatant will then mix with 2.2 volume of chill absolute ethanol, will be incubated at −20 °C for one hour and will be centrifuged at 3500 g for 20min at 4 °C. The pellet containing EPS will be dissolved in sterile water and will be measured by phenol– sulphuric acid method.All the experiments will be replicated for statistical analysis using ANOVA or else. Minor modifications will be done as per the environmental conditions of laboratory

Synergy studies -

For interaction studies, selected extracts of arid zone plants will be mixed with azithromycin and gentamicin separately to test synergistic effect of antibiotic on selected extracts. This assay was performed by a checker board titration method by using 96-well polypropylene microtiter plate. The fractional inhibitory concentration (FIC) index for combinations of antimicrobials was calculated according to the equation:

FIC index = FIC a + F IC b = + A/MIC a + B /MIC b (2)

where A and B are the MICs of extract associated with antibiotic A and extract associated with antibiotic B in the combination, MICA and MICB are the MICs of extract associated with antibiotic A and extract associated with antibiotic B alone, FICA and FICB are the FICs of extract associated with antibiotic A and extract associated with antibiotic B.

EXPECTED OUTCOME OF THE STYUDY

This study will help us to find out the anti-biofilm properties of the selected ethno medicinal properties of the selected plants of arid zone.

Biofilm-associated bacterial infections caused by *Staphylococcus, Bacillus, Pseudomonas, E coli* are found in most of the human diseases. Pathogenic bacterial resistance against antibiotics reduces the effectiveness of many antimicrobial drugs and therapeutic agents. Therefore, alternative ways of reducing biofilms are very necessary to find out.

Various plants found in arid zones having different medicinal properties have a potential to cure these diseases. In this study we will try to find out that extracts of various plants have the ability to inhibit biofilm formation of different bacterial strains against different antibiotics. The anti-adherence and anti-QS compounds present in plants (extracts from leaves, barks, flowers, and roots) help in enhancing the susceptibility of the bacteria and thus in eradicating biofilms.

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